IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

IN RE: '318 PATENT INFRINGEMENT LITIGATION	)	C.A. No. 05-356 (KAJ)
	)	(consolidated)
	)	

NOTICE OF DEPOSITION FOR JOSEPH T. COYLE

To: Steven J. Balick
ASHBY & GEDDES
222 Delaware Avenue, 17th Floor
Wilmington, DE 19899

George F. Pappas
COVINGTON & BURLING
1201 Pennsylvania Avenue, N.W.
Washington, D.C. 20004

Steven P. Berman
Office of General Counsel
Johnson & Johnson
One Johnson & Johnson Plaza
New Brunswick, NJ 08933

PLEASE TAKE NOTICE that, pursuant to Rule 30 of the Federal Rules of Civil Procedure and as indicated in the attached subpoena (Ex. A), Defendants, by and through their attorneys, hereby give notice of their intention, to take the deposition upon oral examination on the date indicated of:

1. Joseph T. Coyle on April 4, 2006.

Dr. Coyle's deposition will commence at **9:00 a.m. EST** on **April 4, 2006**, at the offices of Rose & Associates, 29 Commonwealth Avenue, Boston, MA 02116. The deposition will be taken before a notary public or other officer authorized to administer the oath under law,

and will continue day to day until completed with adjournments as to time and place that may be necessary. The deposition may be recorded by videographic and/or stenographic means.

NOTICE IS FURTHER GIVEN THAT Dr. Coyle is instructed to produce documents, identified in the Rider to the attached subpoena (Ex. A), at the offices of Rose & Associates, Attn: Alan D. Rose, Sr., 29 Commonwealth Avenue, Boston, MA 02116, on or before March 13, 2006.

If counsel for Dr. Coyle or Plaintiffs have any questions regarding this Notice, you are invited to contact any counsel for Defendants to discuss this matter.

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Attorneys for Defendants/Counterclaim-Plaintiffs Mylan Pharmaceuticals Inc. and Mylan Laboratories Inc.

Dated: February 28, 2006

EXHIBIT A

AO88 (Rev. 1/94) Subpoena in a Civil Case

Issued by the

UNITED STATES DISTRICT COURT

DISTRICT OF Massachusetts

In re '318 Patent Infringement Litigation

V.

SUBPOENA IN A CIVIL CASE

Case Number:[1] 05-356-KAJ (consolidated)

(Currently pending in the United States District Court for the District of Delaware)

TO: Joseph T. Coyle, M.D.
Harvard Medical School
25 Shattuck Street
Boston, Massachusetts 02115

☐ YOU ARE COMMANDED to appear in the United States District court at the place, date, and time specified below to testify in the above case.

PLACE OF TESTIMONY	COURTROOM
	DATE AND TIME

☑ YOU ARE COMMANDED to appear at the place, date, and time specified below to testify at the taking of a deposition in the above case.

PLACE OF DEPOSITION	DATE AND TIME
Rose & Associates, 29 Commonwealth Avenue, Boston, Massachusetts 02116	4/4/2006 9:00 am

☑ YOU ARE COMMANDED to produce and permit inspection and copying of the following documents or objects at the place, date, and time specified below (list documents or objects):

See attached Rider and related exhibits

PLACE	DATE AND TIME
Rose & Associates, Attn: Alan D. Rose, Sr., 29 Commonwealth Avenue, Boston, Massachusetts 02116	3/13/2006 10:00 am

☐ YOU ARE COMMANDED to permit inspection of the following premises at the date and time specified below.

PREMISES	DATE AND TIME

Any organization not a party to this suit that is subpoenaed for the taking of a deposition shall designate one or more officers, directors, or managing agents, or other persons who consent to testify on its behalf, and may set forth, for each person designated, the matters on which the person will testify. Federal Rules of Civil Procedure, 30(b)(6).

ISSUING OFFICER'S SIGNATURE AND TITLE (INDICATE IF ATTORNEY FOR PLAINTIFF OR DEFENDANT)	DATE
Amy D. Brody, Attorney for Defendants Mylan Pharmaceuticals Inc. and Mylan Laboratories Inc.	2/23/2006

ISSUING OFFICER'S NAME, ADDRESS AND PHONE NUMBER

Amy D. Brody, Rakoczy Molino Mazzochi Siwik LLP, 6 West Hubbard St., Suite 500, Chicago, IL 60610, 312-222-6344

(See Rule 45, Federal Rules of Civil Procedure, Parts C & D on next page)

[1] If action is pending in district other than district of issuance, state district under case number.

AO88 (Rev. 1/94) Subpoena in a Civil Case

PROOF OF SERVICE

DATE PLACE

SERVED

SERVED ON (PRINT NAME) MANNER OF SERVICE

SERVED BY (PRINT NAME) TITLE

DECLARATION OF SERVER

I declare under penalty of perjury under the laws of the United States of America that the foregoing information contained in the Proof of Service is true and correct.

Executed on _____
DATE

SIGNATURE OF SERVER

ADDRESS OF SERVER

Rule 45, Federal Rules of Civil Procedure, Parts C & D:

(c) PROTECTION OF PERSONS SUBJECT TO SUBPOENAS.

(1) A party or an attorney responsible for the issuance and service of a subpoena shall take reasonable steps to avoid imposing undue burden or expense on a person subject to that subpoena. The court on behalf of which the subpoena was issued shall enforce this duty and impose upon the party or attorney in breach of this duty an appropriate sanction which may include, but is not limited to, lost earnings and reasonable attorney's fee.

(2) (A) A person commanded to produce and permit inspection and copying of designated books, papers, documents or tangible things, or inspection of premises need not appear in person at the place of production or inspection unless commanded to appear for deposition, hearing or trial.

(B) Subject to paragraph (d) (2) of this rule, a person commanded to produce and permit inspection and copying may, within 14 days after service of subpoena or before the time specified for compliance if such time is less than 14 days after service, serve upon the party or attorney designated in the subpoena written objection to inspection or copying of any or all of the designated materials or of the premises. If objection is made, the party serving the subpoena shall not be entitled to inspect and copy materials or inspect the premises except pursuant to an order of the court by which the subpoena was issued. If objection has been made, the party serving the subpoena may, upon notice to the person commanded to produce, move at any time for an order to compel the production. Such an order to comply production shall protect any person who is not a party or an officer of a party from significant expense resulting from the inspection and copying commanded.

(3) (A) On timely motion, the court by which a subpoena was issued shall quash or modify the subpoena if it

(i) fails to allow reasonable time for compliance,
(ii) requires a person who is not a party or an officer of a party to travel to a place more than 100 miles from the place where that person resides, is employed or regularly transacts business in person, except that, subject to the provisions of clause (c) (3) (B) (iii) of this rule, such a person may in order to attend trial be commanded to travel from any such place within the state in which the trial is held, or

(iii) requires disclosure of privileged or other protected matter and no exception or waiver applies, or
(iv) subjects a person to undue burden.

(B) If a subpoena

(i) requires disclosure of a trade secret or other confidential research, development, or commercial information, or
(ii) requires disclosure of an unretained expert's opinion or information not describing specific events or occurrences in dispute and resulting from the expert's study made not at the request of any party, or
(iii) requires a person who is not a party or an officer of a party to incur substantial expense to travel more than 100 miles to attend trial, the court may, to protect a person subject to or affected by the subpoena, quash or modify the subpoena, or, if the party in who behalf the subpoena is issued shows a substantial need for the testimony or material that cannot be otherwise met without undue hardship and assures that the person to whom the subpoena is addressed will be reasonably compensated, the court may order appearance or production only upon specified conditions.

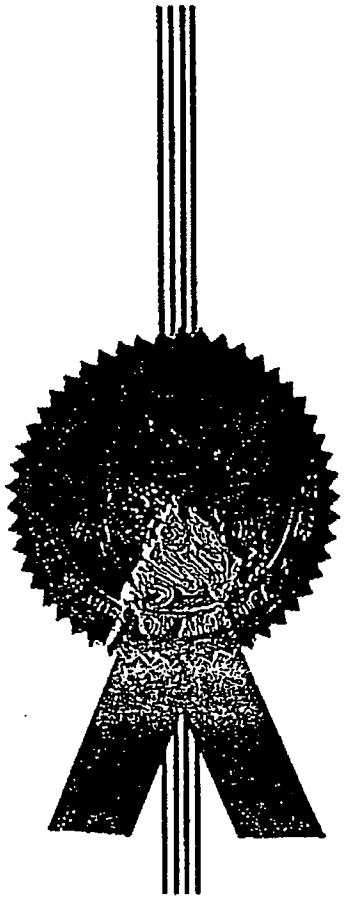
(d) DUTIES IN RESPONDING TO SUBPOENA.

(1) A person responding to a subpoena to produce documents shall produce them as they are kept in the usual course of business or shall organize and label them to correspond with the categories in the demand.

(2) When information subject to a subpoena is withheld on a claim that it is privileged or subject to protection as trial preparation materials, the claim shall be made expressly and shall be supported by a description of the nature of the documents, communications, or things not produced that is sufficient to enable the mmmdemanding party to contest the claim.

Exhibit 1 to Rider to Subpoena directed to Joseph T. Coyle

The United States of America



The Commissioner of Patents and Trademarks

Has received an application for a patent for a new and useful invention. The title and description of the invention are enclosed. The requirements of law have been complied with, and it has been determined that a patent on the invention shall be granted under the law.

Therefore, this

United States Patent

Grants to the person or persons having title to this patent the right to exclude others from making, using or selling the invention throughout the United States of America for the term of seventeen years from the date of this patent, subject to the payment of maintenance fees as provided by law.

Commissioner of Patents and Trademarks

Attest Melvinia Gary

SYN RAZ-0015998

United States Patent [19]

Davis

[11] Patent Number: 4,663,318

[45] Date of Patent: May 5, 1987

[54] **METHOD OF TREATING ALZHEIMER'S DISEASE**

[76] Inventor: Bonnie Davis, 17 Seacrest Dr., Huntington, N.Y. 11743

[21] Appl. No.: 819,141

[22] Filed: Jan. 15, 1986

[51] Int. Cl.[4] A61K 31/55

[52] U.S. Cl. 514/215

[58] Field of Search 514/215

[56] References Cited

PUBLICATIONS

Chem. Abst. (81)–72615z (1974).
Chem. Abst. (86)–115157z (1977).
Horshenson et al. J. Med. Chem. vol. 29, No. 7, 7/86, pp. 1125–1130.
Kendall et al., J. Chem. & Hospital Pharmacol., (1985) 10–327–330.
S. Chaplygina et al., J. of Highest Nervous Activity vol. XXIV 1976 Issue 5, pp. 1–4.
Krause, J. of Highest Nervous Activity, vol. XXII, 1974, Issue 4.

Primary Examiner—Stanley J. Friedman
Attorney, Agent, or Firm—Ladas & Parry

[57] ABSTRACT

Alzheimer's disease may be treated with galanthamine.

7 Claims, No Drawings

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METHOD OF TREATING ALZHEIMER'S DISEASE

GENERAL FIELD OF THE INVENTION

The present invention relates to a novel method of treating Alzheimer's disease and more particularly to a treatment using galanthamine.

BACKGROUND ART

Galanthamine and acid addition salts thereof have, for many years, been known to have anticholinesterase properties. Cozanitis in Anaesthesia 29 163–8 (1974) describes the effect of galanthamine hydrobromide on plasma cortisol of patients receiving relaxant anaesthesia and Cozanitis et al in Acta Anesth. Scand. 24:166–168 (1980) describe the effect of galanthamine on plasma ACTH values during anaethesia. These studies showed an increase in both plasma cortisol and plasma ACTH when galanthamine was administered to patients together with atropine.

Il'yuchenok et al (Chemical Abstracts 70 36296K describe the appearance of θ-rhythm on an electroencephalogram when galanthamine is administered intravenously to rabbits.

Increase in short-term memory in dogs by use of galanthamine is described by Krauz in Chemical Abstracts 81 72615Z.

The antagonistic effect of galanthamine to scopolamine-induced amnesia in rats is described by Chaplygina et al in Chemical Abstracts 86 115157Z, and in Zhurnal Vysshei Nervnoi Deiatelnosti imeni P. Pavlova (MOSKVA) 26:1091-1093, 1976.

Alzheimer's disease, presenile dementia, causes much distress not only to those suffering from the disease, but also those who are close to them. The custodial care of advanced victims of the disease is a tremendous expense to society. At present, there is no effective means of improving the functional status of persons with the disease.

It is an object of the present invention to improve the cognitive function of patients with Alzheimer's disease.

SUMMARY OF THE INVENTION

A method for treating Alzheimer's disease and related dementias which comprises administering to mammals, including humans, an effective Alzheimer's disease cognitively-enhancing amount of galanthamine or a pharmaceutically-acceptable acid addition salt thereof. A radioactively-labelled form of the molecule may also serve as a diagnostic test for Alzheimer's disease.

DETAILED DESCRIPTION OF THE INVENTION

Galanthamine can be administered in any convenient chemical or physcial form. For example, it may be administered as its hydrobromide, hydrochloride, methylsulfate or methiodide.

Galanthamine or its pharmaceutically-acceptable acid addition salts may be administered to a patient suffering from Alzheimer's disease orally or by subcutaneous or intravenous, injection, or intracerebroventricularly by means of an implanted reservoir. It may be necessary to begin at lower doses than are ultimately effective.

Galanthamine and its acid addition salts form crystals. They are in general only sparingly soluble in water at room temperature and so injectible compositions are normally in the form of an aqueous suspension. If necessary, pharmaceutically-acceptable suspension aids may be employed. Typically, such a suspension will be employed at a concentration of 1–50 mg/ml more commonly 5–40 mg/ml, for example, 5–30 mg/ml or 10–40 mg/ml, typically 20–30 mg/ml of galanthamine. Typical dosage rates when administering galanthamine by injection are in the range 5–1,000 mg per day depending upon the patient. For example, divided doses in the range 0.5–5 mg/kg body weight per day may prove useful. Typically, one might administer a dosage of 50–300 mg per day to a patient of a body weight of 40–100 kg, although in appropriate cases such dosages may prove useful for patients having a body weight outside this range. In other cases, dosages as low as 10 mg and as high as 500 mg may be appropriate for persons in this body weight range.

Galanthamine or its pharmaceutically-acceptable acid addition salts may also be administered orally, for example, as an aqueous suspension or a solution in aqueous ethanol or as a solid such as a tablet or capsule. Suspensions or solutions for oral administration are typically of about the same concentration as those used for injections. However, it may be desirable when administering the drug orally to use a higher dosage rate than when administering it by injection. For example, dosages up to 2000 mg per day may be used, such as dosages in the range 100–600 mg per day. In preparing such tablets or capsules, standard tablet or capsulemaking techniques may be employed. The dosage rate of galanthamine or its pharmaceutically-acceptable salt will normally be in the same range as for oral administration of a liquid. If desired, a pharmaceutically-acceptable carrier such as starch or lactose may be used in preparing galanthamine tablets. Capsules may be prepared using soft galatine as the encapsulating agent. If desired, such capsules may be in the form of sustained release capsules wherein the main capsule contains microcapsules of galanthamine which release the contents over a period of several hours thereby maintaining a constant level of galanthamine in the patient's blood stream.

The following test provides a good animal model for Alzheimer's disease in humans: A selective lesion is placed in a subcortical nucleus (nucleus basalis of Meynert) with a resultant cortical cholinergic deficiency, similar in magnitude to that seen in early to moderate stage Alzheimer's disease. Numerous behavioral deficits, including the inability to learn and retain new information, characterizes this lesion. Drugs that can normalize these abnormalities would have a reasonable expectation of efficacyin Alzheimer's disease. Haroutunian, V, Kanof P, Davis, KL: Pharmacological alleviations of cholinergic-lesion-induced memory defects in rats. Life Sciences 37:945–952, 1985.

The following specific formulations may find use in treatment of Alzheimer's disease:

Tablets or capsules containing 5, 10 and 25 mg galanthamine hydrobromide to be taken four times a day, or a sustained-release preparation delivering an equivalent daily dose.

Parenteral solution containing 5 mg/ml.

Liquid formulation for oral administration available in 5 mg/5 ml and 25 mg/5 ml concentration.

There have been reports that galanthamine can cause cardiac arrythmias. In such cases, it may be desirable to

administer galanthamine in conjunction with another drug such as propanthelinbromide to control such arrythmias.

I claim:

1. A method of treating Alzheimer's disease and related dementias which comprises administering to a patient suffering from such a disease a therapeutically effective amount of galanthamine or a pharmaceutically-acceptable acid addition salt thereof.

2. A method according to claim 1, wherein the administration is parenteral at a daily dosage of 5–1,000 mg of galanthamine or a pharmaceutically-acceptable acid addition salt thereof.

3. A method according to claim 2, wherein said dosage rate is 50–300 mg per day.

4. A method according to claim 1, wherein said administration is oral and is in the range 10–2000 mg per day.

5. A method according to claim 4, wherein said dosage rate of 100–600 mg per day.

6. A method according to claim 1, wherein galanthamine is administered at a dosage rate of 0.1 to 4 mg/kg body weight of a patient, parenterally.

7. A method according to claim 1, wherein galanthamine is administered intracerebroventricularly via an implanted reservoir at a dosage rate of 0.01 to 5.0 mg/kg day.

* * * * *

Exhibit 2 to Rider to Subpoena directed to Joseph T. Coyle

Check here if this is a REPLACEMENT of abstract submitted earlier. REMIT $25 for each replacement abstract.

Read all instructions before typing abstract. See *Call for Abstracts* and reverse of this sheet. Complete abstract and all boxes at left and below before making copy.

POSTMARKING: MAY 1, 1987

First (Presenting) Author

Provide full name (no initials), address, and phone numbers *of first author on abstract. You may present only one abstract.*

Joanne Sweeney, Neurotox. Prog., JHU Sch. of Public Health, 615 N. Wolfe St., Balto., MD 21205
Office: (301) 955-8370
Home: (301) 467-1836

Presentation Preference

Check one: [X] poster [ ] slide
Check one to indicate preference if the above is not available. THIS CHOICE WILL BE HONORED:
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Themes and Topics

See list of themes and topics. Indicate below a first and second choice appropriate for programing and publishing your paper.
1st theme title: Neural Basis of Behavior theme letter: I
1st topic title: Learning & Memory; Pharmacology topic number: 108
2nd theme title: Neural Basis of Behavior theme letter: I
2nd topic title: Learning&Memory; Anatomy topic number: 106

Special Requests (e.g., for projection and sequential presentations, days you cannot attend)

Include nonrefundable ABSTRACT HANDLING FEE of $20 payable to the Society for Neuroscience, DRAWN ON A U.S. BANK IN U.S. DOLLARS ONLY.

REVERSAL OF LESION-INDUCED SWIM MAZE DEFICITS WITH A CENTRAL ACETYLCHOLINESTERASE (AChE) INHIBITOR.

L.E. Sweeney, C.F. Höhmann, L.A. Bowersox*, T.H. Moran and J. T. Coyle. Depts. of Environ. Health and Neuroscience. The Johns Hopkins University Schools of Public Health and Hygiene and Medicine, Baltimore, MD 21205.

Destruction of cholinergic neurons of the basal forebrain (BF) which project to neocortex in rodents results in impaired performance on tasks involving working memory. In this study, we examined whether lesions to and pharmacologic manipulation of the central cholinergic system in mice impair performance on a swim maze task. Further, we examined whether this deficit could be reversed by a centrally-acting reversible acetylcholinesterase (AChE) inhibitor, galanthamine hydrobromide (GHB). GHB has an *in vivo* half life of approximately 6 hours, making its effects longer than most previously tested AChE inhibitors.

Adult male Balb/C mice received bilateral ibotenate lesions to BF and were allowed to recover for two weeks. Working memory was assessed in lesioned animals and age-matched controls on a water maze task. The swim tank (72-cm diameter) contained a platform submerged 1 cm below the surface of the opacified water. Mice were placed into each quadrant of the tank, and latency to find the platform was measured. Following acquisition, the position of the platform was changed daily and the new position demonstrated to the animal before latency was measured. After reaching criteria (< 100 sec), mice received saline injections (0.33 ml / kg, i.p.), and on the following day GHB (5 mg/kg, i.p.) one half hour before testing. Another group of mice were trained to criteria and tested with scopolamine (0.8 mg/kg,i.p.), a centrally-acting muscarinic antagonist, or N-methyl scopolamine (0.8 mg/kg), a peripheral antagonist.

Even though no significant differences were noted for acquisition of the task (either days to acquire or latency when the platform remained in one position), a clear distinction could be noted when the platform was moved on consecutive days; mean latency for lesioned animals was 200±63 sec, and mean latency for control animals was 60±6 sec. GHB reduced latency in lesioned animals to 100±17 sec, while latency in control animals increased to 169±20 sec. Within 2 days of the drug treatment, performance in both groups returned to pre-injection levels. Scopolamine injections clearly impaired the animals' performance (latency was 206±44 sec), whereas N-methyl scopolamine, the peripheral muscarinic antagonist did not affect latency (51±3 sec).

We have developed a working memory task for mice which is sensitive to cholinergic interuption, by either lesions to BF or administration of a central muscarinic antagonist. Results suggest that galanthamine can temporarily reverse impaired performance in BF lesioned animals. Further, it appears that optimum levels of acetylcholine (ACh) are necessary for accurate performance of this task; and that either too little or too much ACh is associated with impaired performance. Since galanthamine has a longer half life than many centrally-acting cholinergic drugs, it could be of possible clinical use in patients suffering from central cholinergic losses, for examples, in Alzheimer's Disease.

Supported by grants PO1 HD 19920, 5T32ES 07149 and by the Mc Knight Foundation.

KEY WORDS: (see instructions pg. 4) Do not type on or past blue lines (printer's cut lines) Dimensions of Abstract Form 8 1/16" × 5 5/

1. AChE Inhibitor
2. WORKING MEMORY
3. SWIM MAZE
4. BASAL FOREBRAIN LESION

Signature of Society for Neuroscience member required below. *No member may sign more than one abstract.*

The signing member certifies that any work with human or animal subjects related in this abstract complies with the guiding principles for experimental procedures endorsed by the Society.

Joanne Sweeney — Society for Neuroscience member's signature
JOANNE SWEENEY — Printed or typed name
(301) 955-8370 — Telephone number

Exhibit 3 to Rider to Subpoena directed to Joseph T. Coyle

Pharmacology Biochemistry & Behavior, Vol. 31, pp. 141–147. © Pergamon Press plc, 1988. Printed in the U.S.A. 0091-3057/88 $3.00 + .00

63 B

A Long-Acting Cholinesterase Inhibitor Reverses Spatial Memory Deficits in Mice

JOANNE E. SWEENEY,* CHRISTINE F. HÖHMANN,† TIMOTHY H. MORAN‡ AND JOSEPH T. COYLE*†‡§[1]

*Department of Environmental Health Sciences, Neurotoxicology Division
The Johns Hopkins University School of Public Health
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Received 11 January 1988

SWEENEY, J. E., C. F. HÖHMANN, T. H. MORAN AND J. T. COYLE. *A long-lasting cholinesterase inhibitor reverses spatial memory deficits in mice.* PHARMACOL BIOCHEM BEHAV 31(1) 141–147, 1988.—The effects of the long-acting acetylcholinesterase (AChE) inhibitor, galanthamine, on spatial memory were investigated in mice. Mice received ibotenic acid or sham lesions to the nucleus basalis magnocellularis (nBM). Groups of nBM-lesioned and control mice were then trained on a modified Morris swim maze task. Each mouse was first placed on a platform and then into quadrants of the swim tank in a random order. Time required to find the hidden platform was measured. In different phases of testing, the animal had to find a platform that either remained in the same quadrant (reference memory component) or was moved daily (working memory component). The nBM-lesioned mice took significantly longer to find the platform as compared to controls on the working, but not on the reference, memory component of the task. Galanthamine (5.0 mg/kg, IP), given 3.5 hours before testing, improved performance on the working memory task in nBM-lesioned mice by 70% and strikingly impaired performance in controls. Galanthamine's ability to reverse cognitive deficits induced by nBM lesions and its comparatively long half-life suggest that it may be effective in treating the central cholinergic deficits in Alzheimer's disease patients.

Nucleus basalis lesions Acetylcholinesterase Spatial memory Mice Galanthamine Animal models for Alzheimer's disease

THE important role of central cholinergic neuronal systems in learning and memory has been recognized for a number of years (17). Pharmacological data demonstrate that central muscarinic receptor antagonists impair performance on memory tasks and cause an amnesia-like syndrome in both rodents and primates, including humans (7,18). Conversely, drugs that moderately increase central cholinergic activity enhance performance on memory tasks (5, 20, 38). More recently, lesion studies have assisted in identifying specific cholinergic systems involved in cognitive functions. In rodents and primates, the fronto-parietal cortex and hippocampus receive major cholinergic inputs from basal forebrain projections, the nucleus basalis magnocellularis (nBM) and medial septal area (MSA), respectively (27). Lesions of the nBM and MSA produce behavioral deficits in experimental animals tested on a variety of tasks including passive avoidance, T maze, radial arm maze, stone maze and water maze tasks (21,36).

Reduction of cholinergic markers in neocortex and hippocampus is the neurochemical deficit most commonly associated with Alzheimer's type dementia (AD) (10,13). Furthermore, the severity of cholinergic deficits appears to correlate with the degree of dementia and the density of senile plaques and neurofibrillary tangles in AD patients (14). Accordingly, one pharmacologic strategy for enhancing memory in AD patients has been to increase central cholinergic function by the use of inhibitors of acetylcholinesterase (AChE) to prevent the breakdown of acetylcholine (ACh). Several AChE inhibitors have been used to treat AD including physostigmine, tetrahydroamino acridine (THA), and heptylpyrolol heptylcarbamate (8, 30, 39). Physostigmine, the most widely studied of the AChE inhibitors, reverses scopolamine- and basal forebrain lesion-induced memory deficits in rodents and primates (1, 24, 25). Clinically, physostigmine has been shown to enhance short-term memory in some, but not all, AD patients (30). However, physostigmine suffers from a number of disadvantages which hamper its clinical utility and may account for some of the variable clinical results. The drug exhibits erratic absorption, low bioavailability, an unfavorable toxic to

[1]Requests for reprints should be addressed to Joseph T. Coyle, M.D., Department of Psychiatry, Meyer 4-163, The Johns Hopkins School of Medicine, 600 N. Wolfe St., Baltimore, MD 21205.

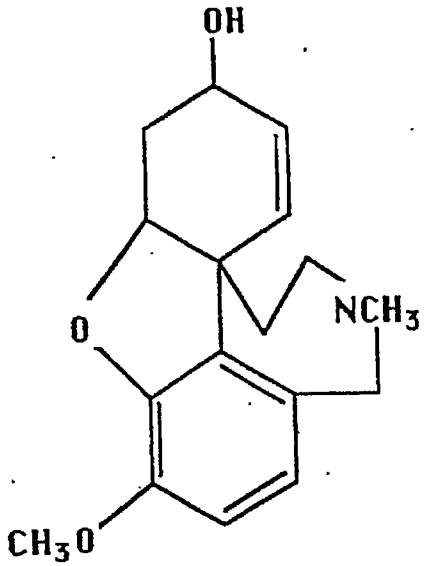

FIG. 1. The structure of galanthamine.

therapeutic ratio, and has a relatively short half-life of 20–30 minutes (16,42).

Galanthamine (Fig. 1) is a centrally-acting competitive AChE inhibitor with a half-life of 4–5 hours in man (12,41). It is a hydrolysis-resistant phenantridine derivative that appears to be more readily absorbed than physostigmine and it possess only moderate toxicity (2). In normal human subjects, galanthamine has been shown to reverse scopolamine-induced impairments including drowsiness, disorientation, delusions and hallucinations (4). In rats, galanthamine antagonized scopolamine-induced amnesia on a conditioned avoidance response (9). To further assess its efficacy in reversing cholinergic deficits, we have examined the ability of this long-acting AChE inhibitor to reverse behavioral deficits in nBM-lesioned mice using a modification of the Morris swim maze task (34).

METHOD

Subjects

Male Balb/cByJ mice (Jackson Laboratories) were 6–8 weeks old at the time of surgery and weighed 26–32 grams at the start of behavioral testing. Mice were housed in groups of 4–5 in standard rodent cages with free access to water and food. Animals were maintained on a 13 hour light/11hour dark cycle with light starting at 7:00 a.m.

Surgery

Bilateral nBM or sham lesions were produced in a two-staged surgical procedure to increase the survival rate of the mice. The first lesion made to the right nucleus, and following a two-week recovery period, a second lesion was made to the contralateral nucleus.

Each mouse was anesthetized with 3% halothane (Ayerst Laboratory Inc.) at a flow of 5–8 liters per minute. The stereotaxic surgical procedure is described elsewhere (28) and summarized here: After the scalp was incised, one hole was drilled anterior to the fronto-nasal suture. The nBM in each mouse was approached by lowering an angled injection needle through the olfactory bulb and moving it in an anterior to posterior and medial to lateral direction. Thus, neither hippocampus nor cortex were directly damaged by the injection. The lesion coordinates were 2.0 mm anterior to the fronto-nasal suture and 1.5 mm lateral to the midline. The needle was lowered to 8.0 mm below the skull surface and then retracted to 7.0 mm where the first of three injections of 0.2 μl ibotenic acid (or saline in sham-treated animals) were made. Two subsequent injections at 6.5 and 6.0 mm were made.

Destruction of the nBM area was produced with the excitotoxin, ibotenic acid, which ablates neuronal perikarya at the site of injection without damaging axons of passage (11). Ibotenic acid was dissolved in a small volume of 1 N NaOH and brought up to a concentration of 10 μg/μl in 0.1 M sodium phosphate buffer, pH 7.4 (if necessary, pH of total solution adjusted to 7.4 with 1 N HCl).

Behavioral testing began following a two-week postoperative recovery period.

Behavioral Testing

Pretraining. Groups of nBM-lesioned, sham-operated, and unlesioned mice were first trained to escape to a platform submerged 1 cm below the surface of 24–26°C milk-opacified water in a 16.5-cm diameter tank. On the first day, the platform was placed against the wall of the tank. Each mouse was placed onto the platform for 30 seconds. The mouse was then placed into a random area of the tank and allowed to swim back to the hidden platform. After climbing onto the platform, the mouse was allowed to rest for 20 seconds. This procedure was repeated five times.

On the second day, training was conducted in a 30-cm diameter tank. On the wall of the tank, above the water line, different black and white patterns were displayed in each quandrant. The platform was placed approximately 5 cm from the wall into the middle of one of the quadrants. The original position of the platform varied (either north, south, east or west) for each animal. First, the animal was placed on the platform for 20 seconds. Then, the mouse was placed sequentially into the middle of each quadrant and allowed to swim back to the platform with a 20 second rest in between each trial.

Training reference memory component. During this phase of training, the platform remained in the same position on each day for a given animal. Since the position in space to which the mouse had to swim did not change, this was considered the reference memory component of this task.

For five days, performance was assessed in a 72-cm diameter tank which contained the same patterns as the smaller tank in each of its quadrants (Fig. 2). The platform was placed approximately 10 cm from the wall in the middle of one of the quadrants. The position of the platform was the same as during pretraining for each animal.

First the animal was placed onto the platform for 20 seconds. Then the animal was placed into the middle of each quadrant, except the one that contained the platform, in a random order. Time to find the hidden platform was measured. If the animals did not find the platform in 120 seconds, it was placed onto the platform for 20 seconds.

Each animal received one training session per day which contained three trials, one from each of the three quadrants (not containing the platform). Training was conducted be-

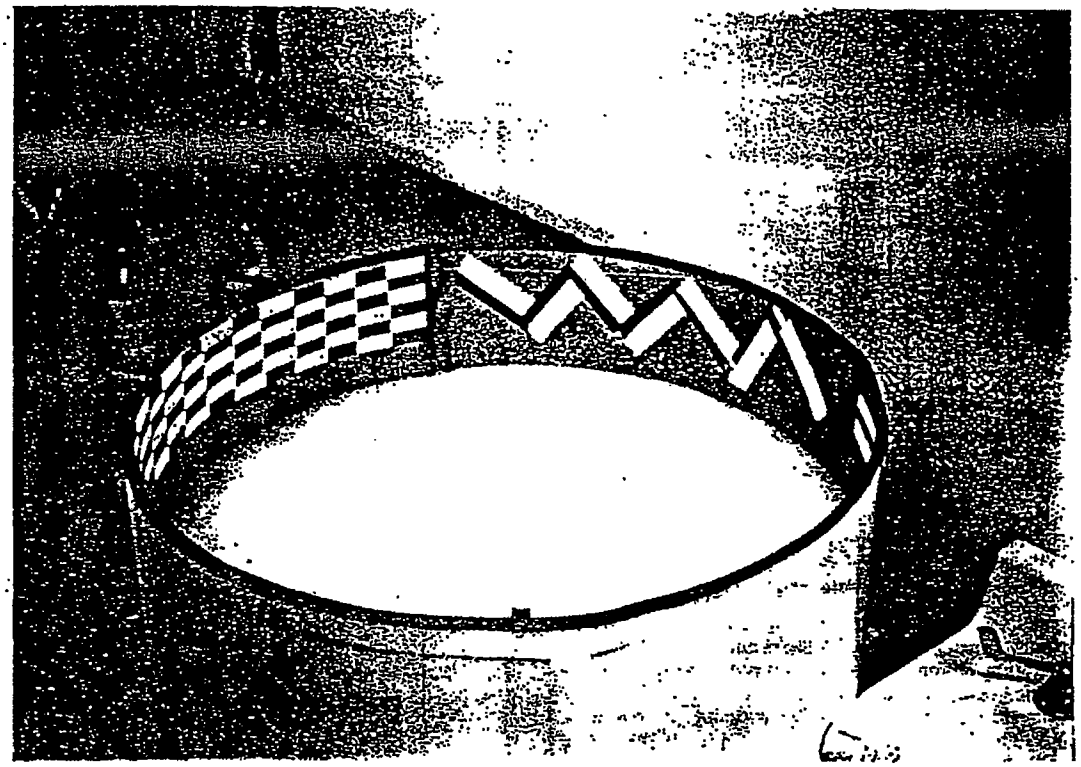

FIG. 2. Photograph of 72-cm diameter swim tank with intramaze cues.

tween 13:00 and 16:00 hours each day. A criterion level was chosen of ≤50 seconds/session for two consecutive days.

Working memory component. In this phase of testing, the position of the platform was changed daily. Since the position in space to which the animal had to swim changed daily, this was considered the working memory component of the task.

First, the animal was placed on the platform for 20 seconds for orientation. Then, as in the reference phase, the animal was placed into the three quadrants of the tank, except the one that contained the platform, in a random sequence. Time to find the hidden platform was measured.

Drug Testing

For the three groups of animals (nBM-lesioned, sham-operated and unlesioned), the effects of galanthamine on performance of the working memory task were assessed. Once an animal had reached a criterion level of performance on the reference component (day 6 after pretraining), each animal received a saline injection (0.1 cc, IP) one hour before the start of the working memory component of the task. The following day (day 7), the three groups received galanthamine (5.0 mg/kg, IP dissolved in saline to 2.0 μg/ml) 3.5 hours before behavioral testing. Each animal was retested 24 hours after administration of the drug.

Biochemistry

Within one week of the completion of behavioral testing, animals were sacrificed for biochemical and histological analyses to examine the efficacy of the lesions and the amounts of cholinergic depletion. Each mouse was decapitated, and the brain rapidly removed onto an ice-cooled metal plate. Tissue samples (approximately 17 mg/hemisphere) were taken from fronto-parietal cortex, not including the cingulate area, and stored at −70°C until the time of assay. The activity of choline acetyltransferase (ChAT) was measured by a modified method of Fonnum (23) using [^{14}C]-Acetyl Coenzyme A (New England Nuclear, 57.2 mCi/mmol; total Acetyl CoA concentration of 500 μM) as substrate. Subsequent separation of the reactants from the product was carried out via an organic ([^{14}C]-acetylcholine extracted into tetraphenyl boron): inorganic (Acetyl CoA into the aqueous phase) separation. Protein was measured according to the method of Lowry (32). All assays were performed in triplicate.

Histology

After removal of samples for biochemical analysis, the remaining brain tissue was fixed by submersion in 4% phosphate buffered formalin, pH 7.4 and 20% sucrose solution (w/v). Frozen brains were sectioned on a sliding microtome into 50 μm coronal sections. Sections through the lesion site were mounted and stained for Nissl substance.

Statistics

Behavioral data were analyzed by a repeated measures analysis of variance (ANOVA); the repeated measure was

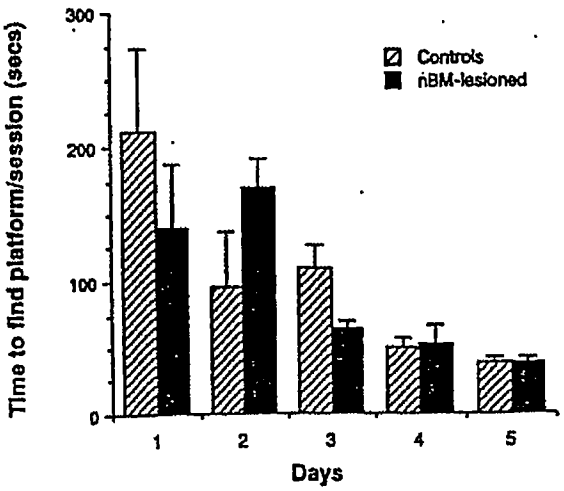

FIG. 3. Acquisition for the reference component of the swim maze task in nBM-lesioned (n=7) and control mice (n=11). The mean time to find the platform/session ±S.E.M. are presented.

the animal's performance at three different times: before treatment with galanthamine, 3.5 hours and 24 hours after its administration. Differences between specific means were assessed using planned *t*-comparisons.

Biochemical data were analyzed using a one-way ANOVA. The data were subjected, post hoc, to Dunnett's test to identify specific differences between the groups.

RESULTS

Behavior

Animals in the nBM-lesioned group that did not have at least a 15% decrease in ChAT activity as compared to controls were excluded from behavioral analyses because it was assumed that the lesion was not successfully located in the nBM region.

Reference memory component. Mean values for the time to find the platform/session were similar for the sham-operated and unlesioned groups. A two-factor ANOVA showed that the two groups did not differ significantly, $F(2,20)=0.79$, $p=0.39$. Therefore, data from these two groups of controls were combined for subsequent behavioral analyses.

Eighty-five percent of the animals in both the nBM-lesioned and control groups achieved criterion levels of performance within five days of the beginning of training (Fig. 3). The two groups performed similarly on the reference memory component of the task. The mean values for the time to find the platform/session for control and lesioned groups did not differ significantly, even though the controls were slightly better than nBM-lesioned animals on days 2 and 4 of the test. On day 5 of the reference component of the test, the mean time to find the platform/session was 38.0±4.0 seconds (±S.E.M.) for nBM-lesioned animals, and was 39.0±4.9 seconds for controls.

Working memory drug treatment. The performance of control and nBM-lesioned mice in the working memory component of behavioral testing depended upon the drug treatment (Fig. 4). There was a highly significant interaction

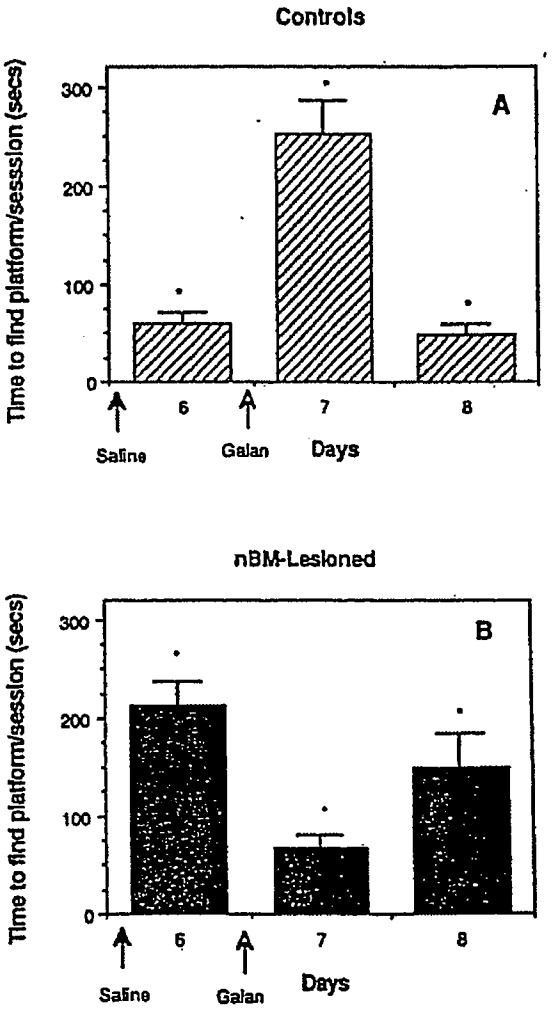

FIG. 4. Effects of saline (0.1 cc, IP, 1 hour before testing) and galanthamine (galan) (5.0 mg/kg, IP, 3.5 hours before testing) on the mean time to find the platform/session ±S.E.M. in control (A) and nBM-lesioned (B) animals on the working memory component of the task. *Repeated measure ANOVA, $F(2,26)=21.2$, $p<0.001$, for interaction effect nBM-lesioned mice vs. controls. Significant differences existed between the groups on day 6, $t(26)=2.92$, $p<0.01$; on day 7, $t(26)=5.44$, $p<0.001$; and on day 8, $t(26)=2.1$, $p<0.05$ (Planned *t*-test). Significant differences also existed before and after the galanthamine treatment in controls, $t(26)=4.65$, $p<0.001$; and nBM-lesioned animals, $t(26)=3.71$, $p=0.001$.

between lesion status and drug treatment, $F(2,26)=21.2$, $p<0.001$. On day 6 of testing, one hour after saline injections, the nBM-lesioned mice demonstrated a clear deficit relative to controls. The mean time to locate the platform/session was 62.3±11.2 seconds for controls and 212.7±25.2 seconds for nBM-lesioned animals ($t=2.92$, $p<0.01$).

Administration of galanthamine (5.0 mg/kg, 3.5 hours before testing) on day 7 significantly decreased the time to find the platform/session in nBM-lesioned animals by 70% to

TABLE 1

ChAT ACTIVITY IN FRONTO-PARIETAL CORTEX DETERMINED 6-8 WEEKS AFTER nBM LESIONS

Groups	ChAT Activity (nmol ACh/mg protein/hr)	% Change
Controls (n=8)	76.9 ± 3.2	—
Sham-Operated (n=3)	73.5 ± 2.5	↓ 0–5%
nBM-Lesioned (n=7)	57.4 ± 2.9*	↓ 15–34%

*$F(5,17)$, $p=0.003$ (one-way ANOVA). nBMs vs. controls $q(10)=4.45$, $p<0.01$; nBMs vs. shams $q(10)=3.47$, $p<0.01$ (Dunnett's test).

67.9±13.4 seconds ($t=3.71$, $p=0.001$). Galanthamine injections produced the opposite effect in controls; the mean time to find the platform/session increased by approximately 400% to 252±35.9 seconds ($t=4.65$, $p<0.001$). The performances of the two groups under the galanthamine treatment were significantly different ($t=5.44$, $p<0.001$).

Twenty-four hours after the drug administration, the mean time to find the platform in controls decreased to 53.7±11.0 seconds, similar to predrug levels. In nBM-lesioned animals, the mean time to find the platform increased to 149.3±35.7 seconds. The two groups were significantly different on day 8 ($t=2.1$, $p<0.05$).

Biochemistry

Levels of ChAT activity in the fronto-parietal cortex were measured 6–8 weeks after surgery in the unlesioned, sham-operated and nBM-lesioned groups (see Table 1). ChAT activity was decreased significantly from 15 to 34% in nBM-lesioned animals as compared to controls. In two nBM-lesioned animals, there was no decrease in ChAT activity in the fronto-parietal cortex. It is interesting to note that in these animals, galanthamine impaired performance of the working memory task, similar to results in control animals.

Histology

In the lesioned animals, the needle tract could be followed from substantia innominata, just lateral to the anterior commissure, to the ventral medial globus pallidus. Gliosis around the needle tract and loss of magnocellular neurons indicated destruction of the nBM.

DISCUSSION

In our study, nBM-lesioned mice demonstrated severe impairment on the working memory, but not the reference memory, component of a swim maze task. Galanthamine (5.0 mg/kg given 3.5 hours before behavioral testing) significantly improved performance of the working memory task in the previously impaired nBM-lesioned animals and significantly impaired performance in controls.

The behavioral task that we have described has clearly distinguishable components assessing performance of both reference and working memory (35). During the acquisition/reference memory phases, the platform remained in the same position each day. Therefore, the rules of performing the task, and the position in space to which the mouse swam, did not change (trial-independent). In contrast, once the platform was moved and the animal was placed on the platform in the new position each day, it was required to remember where the platform was on that particular day and specifically where in space to swim to reach the platform (trial-dependent). The nBM-lesioned animals demonstrated severe impairment on the working, but not the reference, component of the task. In the acquisition/reference memory component, the nBM-lesioned mice demonstrated slightly more variability between days than controls; however, the difference was not statistically significant. Therefore, our study provides indirect evidence that in mice the nBM projection to the cortex is involved primarily in working and not reference memory. Other studies have shown that cholinergic projections to the hippocampus and cortex are involved in working memory, but not reference memory (26,35). However, conflicting data exist, demonstrating the involvement of the nBM projection in reference memory (31,33). These studies were performed in rats and the behavioral tasks were different; therefore, we are unable to compare our results directly to those previously reported.

One drawback to our study was that we were unable to record directly the path length that the mice were swimming. Therefore, we are unable to distinguish at this time whether the working memory deficits in the nBM animals resulted because they were unable to unlearn the first position once the platform was switched, or whether the deficit was in remembering the new position.

The groups of animals (nBM-lesioned and controls) performed similarly on the reference memory component of the task; therefore, the conditions were ideal to test the efficacy of galanthamine on the working memory deficit. Since the groups performed similarly during the first phase of the task, motivation, motor skills, visual acuity and other factors necessary to learn the task were assumed to be similar for the groups. The deficits noted between the groups became clear only on the working memory component of the task, and these specific deficits were reversed by galanthamine. Hence, the improvement seen in the nBM-lesioned animals was most likely related to improvement in memory and not factors, such as improved motor activity.

Other studies further support the hypothesis that we are looking at a memory related phenomenon and not merely alterations in motor activity. In one study, neostigmine, the pheripherally-acting AChE inhibitor, did not improve performance of nBM-lesioned rats on a passive avoidance task, while physostigmine, the centrally-acting compound, did (25). In another study, unilateral ibotenic acid nBM lesions did not affect the speed of swimming in rats (19).

Interestingly, administration of galanthamine severely impaired performance in sham-operated and unlesioned animals at the same dose that it improved performance in nBM-lesioned animals. The dose of galanthamine chosen was based on the highest dose that had produced a consistent behavioral effect in reported literature. It is possible that galanthamine, similar to other AChE inhibitors, exhibits an inverted U-shaped dose-response curve (22,25). In other words, at low doses, these drugs can enhance performance, but higher doses result in impaired performance. Similar findings have been noted in clinical studies in which large doses of physostigmine were given to normal subjects (3,15). Further studies are being carried out to determine the dose-response curve and duration of effect of galanthamine in experimental animals. One explanation for this inverted U-shaped

dose-response curve is that accurate performance of working memory tasks requires optimal levels of ACh at cortical synapses. If this is true, then either insufficient or excessive levels of ACh would impair performance (43). The former would occur if there was a loss of cholinergic input to the cortex after nBM lesions; the latter would occur if ACh breakdown was dramatically inhibited in normals.

ChAT depletion in the cortices in our animals ranged from 15 to 34% which is lower than those reported in many other studies. It is possible that there was recovery of ChAT activity in our animals by the time they were sacrificed 6–8 weeks after the first nBM lesion. Therefore, the ChAT activity reported may not accurately describe ChAT activity at the time of behavioral testing. Several studies report recovery of ChAT activity following unilateral nBM lesions (29,40) and bilateral lesions (37). However, conflicting evidence about the recovery of ChAT activity following bilateral nBM lesions exists (6).

In conclusion, this study provides compelling evidence that galanthamine (5.0 mg/kg, IP) can significantly improve performance of a spatial memory task in nBM-lesioned mice, even when given 3.5 hours before behavioral testing. Clearly, since dysfunction of cholinergic neurons is not the sole cause of the cognitive deficits seen in AD patients, an AChE inhibitor could not be expected to ameliorate all symptoms and restore functions to normal. Nevertheless, these data encourage the expectation that appropriate pharmacological manipulations of the cholinergic system may eventually be developed to alleviate some of the cognitive impairments associated with dementia, such as that seen in Alzheimer's disease.

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CERTIFICATE OF SERVICE

I hereby certify that on the 28th day of February, 2006, I caused a true and correct copy of the foregoing document, **NOTICE OF DEPOSITION FOR JOSEPH T. COYLE**, to be served upon the following counsel of record as indicated below:

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